PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:	Confirmation No: 2174
DEL SOLDATO et al.	Art Unit: 1617
Application No.: 09/147,770	Examiner: R. TRAVERS
Filed: April 28, 1999	Attorney Dkt. No.: 026220-00039

For: NITRIC ESTER DERIVATIVES AND THEIR USE IN GASTROMTESTINAL TUMORS AND OTHER DISEASES

DECLARATION UNDER 37 CFR §1.132

Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Piero Del Soldato hereby declare and state:

I supervised and/or performed the study described in Appendix A hereto. The study demonstrates the enhanced effectivity of nitrooxymethyl phenyl esters of aspirin (NO-Asp-1/2/3) and of flurbiprofen (4-nitrooxy) butyl ester (NO-flurbiprofen) in inhibiting the precancerous cell formation and cancer cell growth as composed to the reference compounds aspirin and flurbiprofen.

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and/or imprisonment under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Name: Piero Del Soldato

Date: October 1, 2003

Enclosure: Appendix A

APPENDIX A

In vivo inhibition of precancerous cell formation and in vitro inhibition of cancer cell growth by the compounds of the invention

EXPERIMENT 1

In vivo assay of the inhibiting activity of precancerous cell formation by the compounds of the invention.

Aberrant crypt foci (ACF) are preneoplastic lesions that have been consistently observed in a number of experimental models of colon carcinogenesis. Moreover, ACF are present in the mucosa of human colon cancer, where they have been suggested to be precursor lesions from which adenomas and carcinomas develop.

Since ACF express mutations in the *apc* gene and the *ras* oncogene, these lesions have been considered early markers of colon cancer development.

The compounds assayed in this test were the following:

- 2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester. The compound was prepared according to ex. 1 of WO 00/44705 (NO-Asp-1).
- Aspirin.

Male wistar rats (weight 200-250 g) were randomised into n. 3 groups of 8-9 animals each at the beginning of the experiment.

Colonic adenocarcinoma was induced by sequential treatment (i.v. injection) with trinitrobenzene sulfonic acid (TNBS) and azoxymethane (AOM) according to the experimental model of D'argenio et Al., Gastroenterology 110, 1727-1734 1996.

The test drugs were dissolved in DMSO diluted in 0.5% carboxymethylcellulose.

The treated groups were administered, respectively, with a daily dose of aspirin (10 mg/Kg) and with an equimolar dose (18 mg/Kg) of NO-Asp-1.

The control group was administered with the vehicle.

The treatment with TNBS and AOM lasted 28 days. Oral drug administration was continued. Six weeks after the end of the experiment the animals were sacrificed by an overdose of

pentobarbital. Laparatomy was then performed with the whole colon excision.

After flushing with 0.9% saline, the colon was tied at both ends with a silk suture and filled with 10% phosphate-buffered formalin (pH 7.4). After 2 hours the colon specimen was opened by cutting along the mesenteric border and pinned flat. Colon mucosa was then dipped into formalin.

After this treatment and rinsing from formalin, tissues were stained with 0.2% methylene blue in 0.9% saline. After 15 minutes the tissues were recovered and the number of ACF in the entire colon specimen, using a dissecting microscope at 40χ magnification. ACF were clearly identified as abnormally dilated crypts, with multiple adjacent crypts, often appearing to be contiguous.

Administration of TNBS and AOM in the control group resulted in the development of widespread precancerous cells formation in the distal colon.

Data are reported in Table 1, wherein the number of ACF cells developed in the colon of the animals of the control group was assumed to be 100%. The Table shows that NO-Asp-1 is more effective than aspirin in preventing colon neoplastic lesion.

Table 1

In vivo- inhibition of precancerous cell formation by NO-Asp-1 and aspirin in an experimental model of colon Adenocarcinoma		
Compound	Dose (mg/Kg)	Precancerous cell Number (% to the control group)
Vehicle	-	100
NO-Asp-1	18	15
Aspirin	10	40

EXPERIMENTS 2-A) and 2-B)

In vitro assay of the antiproliferative activity of the compounds of the invention in cancerous cells.

Experiment 2-A)

Human adenocarcinoma (HT29) cells taken from colon affected by cancerous process were transferred into plates with 24 wells containing a cellular culture medium formed by 10% of foetal bovine serum, penicillin (50 U/ml), streptomycin (50 mg/ml) and PEG 400 (polyethylenglycol).

The compounds tested have been the following:

- 2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester. The compound was prepared according to ex. 1 of WO 00/44705 (NO-Asp-1).
- 2-(acetyloxy)benzoic acid 4-(nitrooxymethyl)phenyl ester The compound was prepared according to ex. 3 of WO 00/44705 (NO-Asp-2).
- 2-(acetyloxy)benzoic acid 2-(nitrooxymethyl)phenyl ester The compound was prepared according to ex. 2 of WO 00/44705 (NO-Asp-3).
- Aspirin.

After 24 hours a portion of the plates was inoculated with the tested compounds dissolved in the carrier (PEG 400). 96 hours after the inoculation of the compounds the cellular growth was measured by haemocytometer. The results, reported in Table 2, are expressed as percentage of the cellular proliferation with respect to the controls.

The obtained results show that the compounds of the invention are much more effective in inhibiting the proliferation of the cancerous cells with respect to the corresponding native compound.

Table 2

Activity in vitro on the proliferation of cancerous cells		
Treatment	Concentration (μM)	Proliferation %
Vehicle	-	100
Aspirin	500	100
NO-Asp-1	300	40
NO-Asp-2	10	0
NO-Asp-3	20	50

Experiment 2-B)

In this experiment flurbiprofen (4-nitrooxy)butyl ester (NO-flurbiprofen) and flurbiprofen, as reference compound, were used.

Flurbiprofen (4-nitrooxy)butyl ester was obtained according to Ex. 1 of WO 94/12463.

HT-29 and HCT-15 human colon adenocarcinoma cell lines (American Type Culture Collection) were grown as monolayers in McCoy 5A medium and RPMI 1640 respectively, and supplemented with 10% foetal calf serum (FCS), penicillin (50 U/ml) and streptomycin (50 mg/ml). Cells were seeded at a density of 1,5 million cells/100 cm^2 culture dish and incubated at 37°C in 5% CO_2 and 90% relative humidity. Single-cell suspensions were obtained by tripsinization (0.05% trypsin/EDTA), and cells were counted using a hemacytometer.

Viability was determined by the tryptan blue dye exclusion method.

The compounds under test were dissolved in dimethyl sulfoxide (DMSO) solutions. All compounds were added to the culture medium before plating. Final DMSO concentration was adjusted in all media to 1% w/v.

A control group was run by adding the the cells a same quantity of DMSO.

NO-flurbiprofen reduced the number of HT-29 cells in the culture more effectively than flurbiprofen. Similar results were obtained with HCT-15 cells.

48 hours after incubation with the compounds, the cells were counted using a hemacytometer. Table 3 reports the results obtained, expressed as percentage of cellular growth with respect to the control group.

Table 3

Activity in vitro on the proliferation of cancerous cells		
Treatment	Concentration (μM)	Growth at 48 hours
Vehicle	-	100
Flurbiprofen	500	55
NO-Flurbiprofen	500	30

N, M, M & O Docket No. _____ NIKAIDO, MARMELSTEIN, MURRAY & ORAM LLP

Declaration For U.S. Patent Application

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below my name.
I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
(Insert Title) NITRIC ESTER DERIVATIVES AND THEIR USE IN URINARY INCONTINENCE AND OTHER DISEASES.
the specification of which is attached hereto unless the following box is checked:

☐ was filed on Sept. 2, 1997 as United States Application Number or PCT International Application Number PCT/EP97/04774 and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56.
I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International Application having a filing date before that of the application(s) for which priority is claimed:

(List prior foreign applications. See note A on back of this page)	(Number)	(Country)	(Day/Month/Year Filed)	Priority Claimed
	MI96A001821	ITALY	4 September 1996	☒ Yes ☐ No
				☐ Yes ☐ No
				☐ Yes ☐ No

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)

(See Note B on back of this page) ☐ See attached list for additional prior foreign or provisional applications.

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) (U.S. or PCT) in the manner provided by the first paragraph of 35, U.S.C. §112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(List prior U.S. Applications or PCT International applications designating the U.S.)	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

And I hereby appoint as principal attorneys David T. Nikaido, Reg. No. 22,663; Charles M. Marmelstein, Reg. No. 25,895; George E. Oram, Jr., Reg. No. 27,931; Robert B. Murray, Reg. No. 22,980; Martin S. Postman, Reg. No. 18,570; E. Marcie Emas, Reg. No. 32,131; Douglas H. Goldhush, Reg. No. 33,125; Kevin C. Brown, Reg. No. 32,402; Monica Chin Kitts, Reg. No. 36,105; Richard J. Berman, Reg. No. 39,107; and James A. Poulos, III, Reg. No. 31,714.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(See Note C on back of this page)

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